

# Elevated CO<sub>2</sub> levels affect development, motility, and fertility and extend life span in *Caenorhabditis elegans*

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**Hypercapnia (high CO<sub>2</sub> levels) occurs in a number of lung diseases and it is associated with worse outcomes in patients with chronic obstructive lung disease (COPD). However, it is largely unknown how hypercapnia is sensed and responds in nonneuronal cells. Here, we used *C. elegans* to study the response to nonanesthetic CO<sub>2</sub> levels and show that levels exceeding 9% induce aberrant motility that is accompanied by age-dependent deterioration of body muscle organization, slowed development, reduced fertility and increased life span. These effects occur independently of the IGF-R, dietary restriction, egg laying or mitochondrial-induced aging pathways. Transcriptional profiling analysis shows specific and dynamic changes in gene expression after 1, 6, or 72 h of exposure to 19% CO<sub>2</sub> including increased transcription of several 7-transmembrane domain and innate immunity genes and a reduction in transcription of many of the MSP genes. Together, these results suggest specific physiological and molecular responses to hypercapnia, which appear to be independent of early heat shock and HIF mediated pathways.**

aging | gene expression | hypercapnia | muscle deterioration | physiology

The internal environment of a living organism self-regulates CO<sub>2</sub>/H<sup>+</sup>. In mammals the lungs are the organs that dispose of excess CO<sub>2</sub> produced in the different tissues by adjusting the ventilatory pattern. Hypercapnia occurs in a number of lung disease states and usually reflects hypoventilation inadequate gas exchange. Some investigators have proposed that high CO<sub>2</sub> levels had beneficial effects in models of acute lung injury and proposed the term “permissive hypercapnia” and even “therapeutic hypercapnia” (1, 2). However, more recent studies have suggested that high pCO<sub>2</sub> can cause oxidative stress in the lung, and injury (3). More recently it has been reported that in rat lungs and human epithelial cells, high pCO<sub>2</sub> decreased alveolar fluid clearance independently of pH and ROS (4, 5). In some reports it has been shown that CO<sub>2</sub> uptake involves the aquaporin and RH1 channels (6, 7). In red blood cells, the RH1 complex also functions as an ammonium transporter (8). The sensing of CO<sub>2</sub> levels in the brain involves CO<sub>2</sub>/H<sup>+</sup> chemoreceptors. CO<sub>2</sub> chemoreceptors were also identified in the central and peripheral nervous system and pulmonary vascular tissues (9, 10). However, very little is known about what senses the CO<sub>2</sub> levels and how these tissues respond to hypercapnia. The effect of low pH (acidosis) in kidney and lung cells can be altogether separated from that of high CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> (4, 11).

The genetically tractable model organism, *C. elegans*, is a very powerful system in which to investigate cellular sensing and response to CO<sub>2</sub>. Despite being an invertebrate, *C. elegans* has differentiated tissues including hypodermis, epidermis, muscle, nervous system and others. Also demonstrated is the importance of evolutionary conserved genes in studying diseases and specific biological processes including hypoxia, longevity, and others.

Recent studies show an acute avoidance of *C. elegans* from CO<sub>2</sub> levels as small as 1% (12, 13). We have chosen to expose *C. elegans* to nonanesthetic elevated levels of CO<sub>2</sub> (14) to describe the physiological and cellular effects of hypercapnia and to determine the molecular pathways that mediate cellular response to hypercapnia at the level of a whole organism.

## Results

**Growing *Caenorhabditis elegans* in Air-Containing 9–19% CO<sub>2</sub> Slows Down Development and Causes Reduced Fertility.** Wild-type *C. elegans* (N2) were maintained on NGM plates under standard conditions (15). Approximately 30 embryos/plate were placed under the following atmospheric conditions: normal air, 5%, 9%, 15%, or 19% CO<sub>2</sub> in air at 20 °C or in the same CO<sub>2</sub> concentrations at 25 °C. Each experiment was repeated 2–5 times. Development was monitored daily until the adult stage. During the egg-laying period, single animals were transferred daily to a fresh plate and the total brood size was scored (Fig. 1 and Table S1). More than 90% of the animals grown for 24 h or 48 h at 25 °C in normal air or in air containing 5% CO<sub>2</sub> reached the L2 or adult stages, respectively. In contrast, after 24 h at 25 °C in 9% CO<sub>2</sub> the animals only reached either L1 or early L2 stages, and after 48 h they only reached late L4 or early adult stages. Growing the animals in 15% or 19% CO<sub>2</sub> in air caused additional delay in development (Table S1); after 24 h all animals were at the L1 stage and after 48 h they were either at the L3 or L4 stages. Even after 72 h at 25 °C in 19% CO<sub>2</sub>, >90% of the animals were still at the L4 stage. Similarly, after 72 h at 20 °C in normal air or in 5% CO<sub>2</sub> all animals were adults. In contrast, animals in 15% or 19% CO<sub>2</sub> were at L4 or at L3/early L4, respectively. DIC microscopic analysis and DAPI staining of nuclei showed that there were no apparent developmental defects and the adult animals looked normal, suggesting that growing *C. elegans* in air containing CO<sub>2</sub> levels of 9% and above causes a delay in development.

Animals grown in normal air at 25 °C laid an average of 163 eggs. Growing animals in 5% CO<sub>2</sub> caused a small reduction to an average of 140 eggs (Fig. 1A). Growing the animals at 25 °C in 9% CO<sub>2</sub> reduced the number of eggs to 51% compared with wild type, whereas growth in 15% or 19% CO<sub>2</sub> further reduced egg laying to only 20% or 11%, respectively (Fig. 1A). The average egg laying of animals grown in air at 20 °C was 250. In 15% CO<sub>2</sub> or 19% CO<sub>2</sub> the average egg laying was reduced to 47%

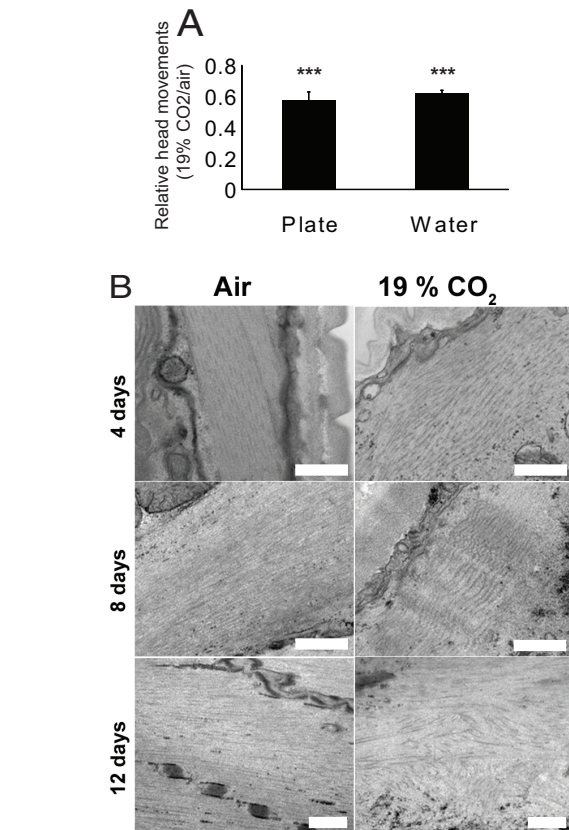
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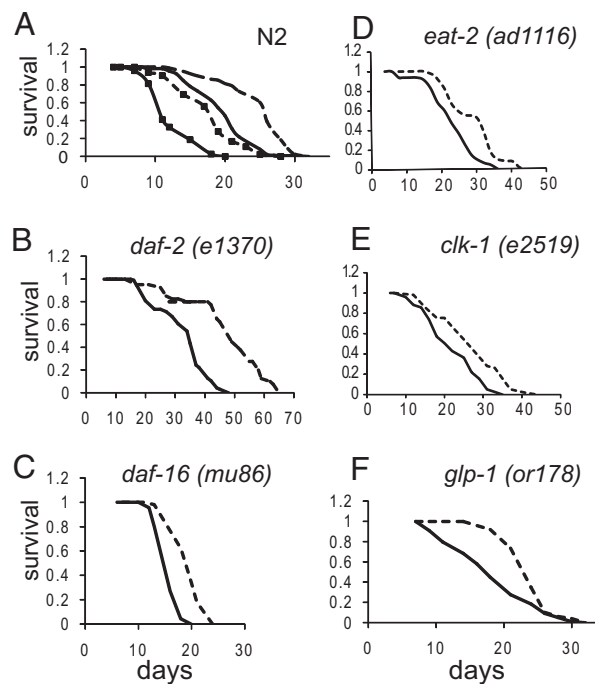




**Fig. 2.** Growth in air containing 19% CO<sub>2</sub> reduces motility and affects muscle morphology. (A) The average number of head movements of wild-type (N2) animals at the L4 stage grown in air ( $n = 22$ ) or in air containing 19% CO<sub>2</sub> at 20 °C was scored either on NGM plates (Left,  $n = 21$ ) or in a water drop (Right,  $n = 21$ ). All measurements were performed after the animals were removed from the CO<sub>2</sub> chamber. The number of head movements/minute was divided by the average number of head movement of animals grown in air. (B) Thin-section electron micrographs demonstrating the gradual deterioration of body muscles in animals grown for 4, 8, or 12 days in air containing 19% CO<sub>2</sub> at 20 °C. Muscle morphology was normal in animals grown in air. The muscle of animals grown in air containing 19% CO<sub>2</sub> had deteriorated already at day 4 and muscle filaments were further disorganized at days 8 and 12. (Scale bars, 500 nm.)

biosynthesis pathway required for normal aging (19), in air containing 19% CO<sub>2</sub> at 20 °C increased mean life span from 22.0 days to 26.9 days (Fig. 3E). Thus, the hypercapnia-induced increase in mean life span could be independent of both diet restriction and mitochondria-induced aging pathways. Furthermore, the hypercapnia-induced increase in life span is probably independent of the hypercapnia-induced reduction in egg laying, because the average life span of the temperature sensitive *glp-1(or178)* animals, which do not produce any eggs when grown in 19% CO<sub>2</sub> at 25 °C (20), increased from 18.6 to 24.1 days (Fig. 3F).

**Exposure of *C. elegans* to Elevated CO<sub>2</sub> Levels Causes Major Changes in Gene Expression.** The many and complex phenotypes detected in animals grown in hypercapnia conditions (see above) are accompanied with changes in the expression of specific genes. To identify the genes that respond to hypercapnia, we performed transcription profiling analyses of *C. elegans* exposed to air containing 19% CO<sub>2</sub> at 20 °C at 3 time points: 1, 6 and 72 h and analyzed the changes in immediate early genes, early genes and genes that respond to chronic exposure to elevated CO<sub>2</sub> levels.



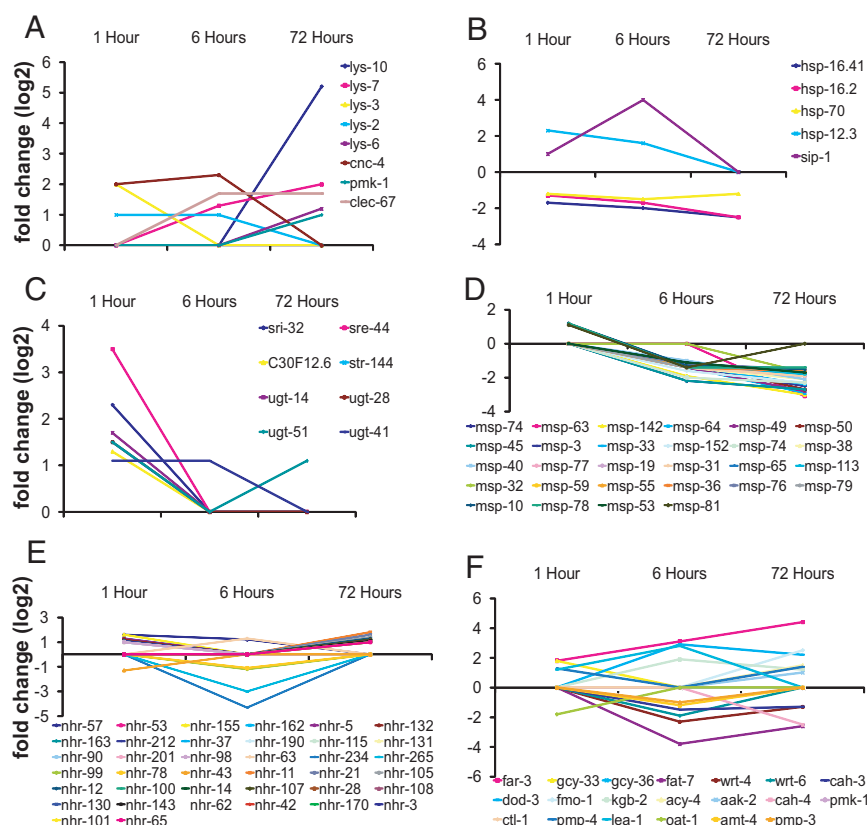
**Fig. 3.** Continuous exposure to air containing 19% CO<sub>2</sub> extends average life span. Survival plots of wild-type (N2) animals, or animals mutated in *daf-2*, *daf-16*, *eat-2*, *clk-1* or *glp-1* genes. Animals were grown at 20 °C in normal air (continuous line in A–E) or in air containing 19% CO<sub>2</sub> (broken lines in A–E), or at 25 °C in normal air (continuous line in F and continuous line with squares in A) or in air containing 19% CO<sub>2</sub> (broken line in F or broken line with squares in A). The average life span is shown in Table S2. There was a significant extension in life span ( $P < 0.0001$ ) in all animals grown in air containing 19% CO<sub>2</sub>.

RNA was isolated and hybridized to GeneArray chips (Affymetrix). The results of transcription profiling analyses appear in [Table S3](#), [Table S4](#), and [Table S5](#). The chip results were verified by RT-qPCR analyses performed on 2 independent RNA isolations. In almost all tested cases (27/33) RT-qPCR and GeneArray results were consistent with each other ([Fig. S2](#)). Because it frequently happens in validation experiments, for the few unvalidated genes their Affymetrix probe sets do not correspond to the same sequences detected by RT-qPCR and it is therefore possible that both methods actually represent different transcripts of the same genes due to alternative splicing for example.

After 1 h of exposure to hypercapnia 429 genes were up-regulated and 59 genes were down-regulated at least 2-fold. After 6 h and 72 h of growth in 19% CO<sub>2</sub>, there were 374/771 up-regulated and 283/657 down-regulated genes, respectively. Among the genes that were up-regulated after 1 h there were 8 genes of the 7-transmembrane domain genes, which could be receptors for chemical messengers (21), 37 genes of the nuclear hormone receptor family, which are involved in transcriptional regulation, 3 genes of the E3 ubiquitin ligase family, which are involved in protein degradation and 8 genes that are involved directly or indirectly in innate immune response (Fig. 4 and Table S6).

Although most up-regulated genes regained their normal levels of expression after 6 or 72 h of growth in 19% CO<sub>2</sub>, few genes remained up-regulated in all 3 time points including *hsp-12.3*, *far-3*, *lea-1* and others (Table S6). Interestingly, although *hsp-12.3* was up-regulated, most of the heat shock genes were down-regulated or remained unchanged. Among the down-regulated genes after 6 h of exposure to 19% CO<sub>2</sub> were the major





**Fig. 4.** Hypercapnia induces change in gene expression. Fold change in log<sub>2</sub> scale of gene expression during 1, 6 or 72 h of exposure to air containing 19% CO<sub>2</sub> of innate immunity (A), heat shock (B), 7 transmembrane domain (C), major sperm proteins (D), nuclear hormone receptor (E), and several other genes of interest (F). The data are taken from [Table S3](#), [Table S4](#), [Table S5](#), and [Table S6](#).

sperm proteins (MSP) and among the down-regulated genes after 72 h of exposure to 19% CO<sub>2</sub> were the carbonic anhydrase genes *cah-3* and *cah-4*.

**Response to High CO<sub>2</sub> Levels Is Independent of Aquaporin and Rhesus Genes.** To test genes that are potentially involved in the transport of CO<sub>2</sub>, we used strains that are each homozygous for a deletion in aquaporin or rhesus gene. We asked whether under hypercapnia conditions the deletion of the gene further delays development and reduces the number of laid eggs or whether it rescues the hypercapnia-induced phenotypes.

A recent report suggests that the aquaporin 1 gene is involved in transporting CO<sub>2</sub> across membranes (22). AQP-2 is one of the *C. elegans* aquaporin 1 orthologs. The *aqp-2(ok2159)* strain, which is homozygous for a deletion in the *aqp-2* gene failed to lay eggs and developed slower when placed in 19% CO<sub>2</sub> at 20 °C (Fig. 5A). *C. elegans* contains 2 rhesus genes: *rhr-1* and *rhr-2*. When grown in 15% CO<sub>2</sub> (Fig. 5) or at 19% (data not shown) at 20 °C the *rhr-1(ok432)* or *rhr-2(ok403)*, the number of eggs laid was significantly reduced for both strains as compared with wild-type animals. These data suggest that the response to hypercapnia is independent of these genes.

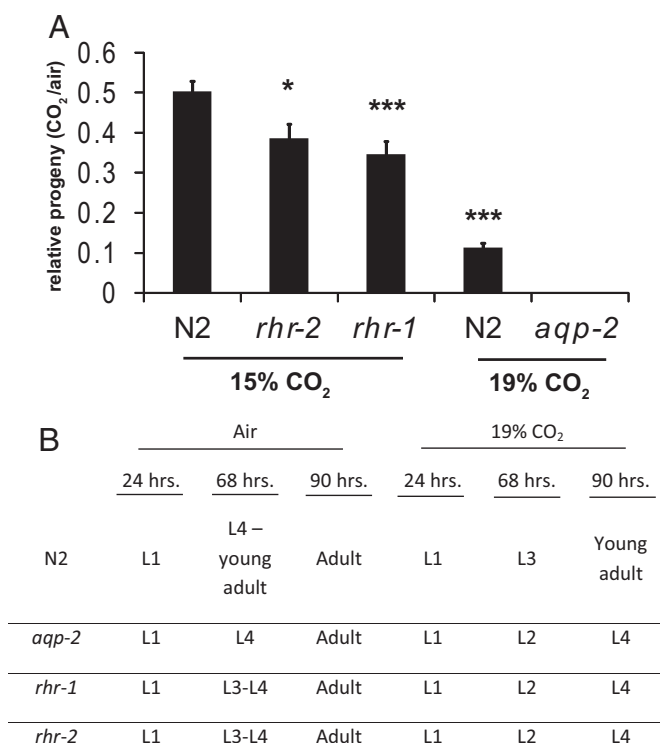
## Discussion

**Exposure of *C. elegans* to Hypercapnia Reduces the Rate of Development and Causes Lower Fertility, Reduced Motility, and Extension of Life Span.** The CO<sub>2</sub> levels in humans arterial blood are normally maintained at  $\approx 5.0\%$  CO<sub>2</sub>. Hypercapnia may occur in a number of lung disease states such as acute respiratory failure, asthma, hypoventilation, sleep apnea and COPD and usually reflects inadequate gas exchange (23). There is still controversy of

whether hypercapnia can be beneficial in acute states or not, however, in patients with COPD it has been associated with worse outcomes. Despite its health and scientific importance, there are relatively few studies addressing the molecular sensing and response of nonexcitable cells to hypercapnia. Here, we used *C. elegans* as a model organism to study the effects caused by hypercapnia in the context of a whole multicellular animal. Our results show that growing *C. elegans* in CO<sub>2</sub> levels of 9%, which are commonly observed in patients with pulmonary diseases, causes lower fertility and a delay in development. Increasing the CO<sub>2</sub> levels to 15% or 19%, revealed similar phenotypes, but further reduction in egg laying and development. The delay in development was not accompanied by morphological defects; the adult animals looked normal. This phenomenon resembles the delay in growth of *C. elegans* at a lower temperature. However, growing *C. elegans* at lower temperature increases fertility, whereas hypercapnia reduces fertility. These data suggest that these 2 environmental conditions probably affect different signaling pathways.

O<sub>2</sub> levels in air containing 19% CO<sub>2</sub> are reduced from 21% to  $\approx 17\%$  as measured by a Clark type O<sub>2</sub> electrode. However, the hypercapnia phenotypes cannot be due to the lower oxygen level because *C. elegans* can maintain the same metabolic rate at 3.6% O<sub>2</sub> as at 21% O<sub>2</sub> and the optimal O<sub>2</sub> levels are between 5–12% (24, 25).

Growing *C. elegans* for short periods under hypercapnic conditions had only minor effects on both development and laying egg, whereas continuous exposure to hypercapnia had more severe effects. These data suggest that the animals can recover quickly from most unhealthy effects of shorter exposure to hypercapnia, the effects of hypercapnia on development and



**Fig. 5.** *Aqp-2*, *rhr-1* and *rhr-2* affect the hypercapnia-induced egg laying. (A) Animals homozygous for a deletion in a specific gene were maintained in air containing 15% or 19% CO<sub>2</sub> and the number of laid eggs was scored and divided by the number of eggs laid by animals of the same genetic background grown in normal air. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.0005$ . (B) The effect of mutations in *aqp-2*, *rhr-1*, and *rhr-2* on the rate of development in normal air and in air containing 19% CO<sub>2</sub>.

on egg laying occur only at specific time points in development, or both. Supporting the latter possibility is the strong effect of growing L4 animals in air containing 19% CO<sub>2</sub>.

Chronic exposure to hypercapnia also caused motility defects that were accompanied by deterioration in muscle morphology. Hypercapnia-induced changes in muscle morphology became more severe with aging. Interestingly, muscle weakness is also observed in patients with COPD, suggesting that muscle deterioration in humans could be related to hypercapnia by yet unknown mechanisms.

Another global effect of hypercapnia is the significant increase in longevity of *C. elegans* grown in 19% CO<sub>2</sub>, which probably occurs independently of the common longevity pathways of the insulin receptor signaling pathway (*daf-2* and *daf-16*), the sterility pathway (*glp-1*), the diet restriction pathway (*eat-2*) or the ubiquinone biosynthesis pathway (*clk-1*). The hypercapnia effect might not be regulated by the diet deprivation-induced life span pathway (26, 27) because it depends on *eat-2* and the life span of *eat-2* animals was significantly increased in 19% CO<sub>2</sub>. Finding the aging pathway affected by hypercapnia is a major goal for future studies.

**Exposure of *C. elegans* to High CO<sub>2</sub> Levels Affects Gene Expression.** Growing *C. elegans* in air containing 19% CO<sub>2</sub> caused dynamic changes in transcription profiles. We have identified a large number of genes that already after 1 h of exposure had a change of 2-fold or greater in their level of transcription. Although some genes remained up-regulated or down-regulated, after 6 h most genes went back to their baseline expression levels. It was also interesting to note that after 72 h of exposure to 19% CO<sub>2</sub>, >6%

of the total genes were either up-regulated or down-regulated. The chip microarray results probably represent a true difference in gene activity, because the real time quantitative PCR, which was performed on independent preparations of RNA, gave results that were consistent with the microarray results.

Many of the known genes that were up-regulated after 1 h of exposure to air containing 19% CO<sub>2</sub> are probably involved in coordinating the initial response of the animal to hypercapnia. The 7 transmembrane domain genes, which include the G protein coupling chemoreceptor *sre-44*, could aid sensing changes in CO<sub>2</sub> levels. The change in many nuclear hormone receptor genes is probably involved in the activation or repression of many genes required to adjust the cellular metabolism.

The change in expression levels of many genes after exposure to air containing 19% CO<sub>2</sub> suggests that hypercapnia is a significant stress to the animal. Surprisingly, although most known heat shock genes were either down-regulated or unchanged, only the *hsp-12.3* gene remained up-regulated throughout the entire period of exposure to 19% CO<sub>2</sub> and only the *sip-1* gene was up-regulated after exposure to 19% CO<sub>2</sub> for 1 or 6 h. In addition, the change in gene expression was completely different from that of exposure to hypoxia (28), suggesting a very different response to elevated levels of CO<sub>2</sub>. Determining the roles of these genes in sensing and/or response to hypercapnia, alone or in combination is a goal for future studies. A surprise finding was the downregulation of many of the MSP genes after 6 h of exposure to 19% CO<sub>2</sub>. We cannot, however, exclude the possibility of a slight change in the developmental rate, which may account for this difference.

Mutant in the rhesus and aquaporin genes show a stronger effect of CO<sub>2</sub> than does wild type (N2), suggesting that these genes may play roles in resisting to hypercapnia. This is in line with previous reports in *C. elegans* or in other organisms (7, 29) and suggests that their role in allowing CO<sub>2</sub> to enter cells is evolutionarily conserved.

## Materials and Methods

**Maintenance of Strains and Growth in CO<sub>2</sub> Chamber.** *C. elegans* strains were handled as described in ref. 15. N2, *aqp-2(ok2159)*, *rhr-1(ok432)*, *rhr-2(ok403)*, *daf-2(e1370)*, *daf-16(mu86)*, *glp-1(or178)*, *eat-2(ad1116)*, *clk-1(e2519)* were obtained from the *C. elegans* Genetic Center. These mutants are out-crossed to the N2 animals. DYNAMENT CO<sub>2</sub> controller with a mini infrared sensor (0–20% CO<sub>2</sub>) was connected to a sealed Perspex incubator. CO<sub>2</sub> was flowed to the incubator via the controller until reaching the desired level and was balanced using air.

**Progeny Number, Life Span, and Locomotion.** Synchronized embryos were transferred to air containing 19% CO<sub>2</sub> and allowed to reach adulthood. Each experiment contained 30 plates and each plate contained 1 animal. Aging experiments were performed as described in ref. 30, gravid worms were allowed to lay eggs for 6–24 h at 20 °C in normal air conditions and embryos were transferred to 19% CO<sub>2</sub> (day 1). The aging experiments with the *glp-1* TS mutant were at 25 °C; the *glp-1* worms were allowed to lay eggs for ≈6 h at 20 °C and only then were transferred to either air or 19% CO<sub>2</sub> at 25 °C.

Animals were considered dead when they did not respond to prodding with a platinum wire. *P* values were calculated using the log-rank method. For the motility measurements, individuals L4 animals were transferred to NGM plates seeded with OP50 or to a drop of M9 and filmed with a webcam. Body bends were counted every time the part of the worm just behind the pharynx reached a maximum bend in the opposite direction from the bend last counted.

**ATP Measurements.** Young adult worms from five 90-mm NGM plates were collected and aliquots of 100 μL were prepared and stored at –70 °C until used. For in vitro ATP measurement 100 μL of worms in 1 mL of DDW were transferred to boiling water for 15' and then centrifuged for 5' at 14000 rpm. Serial dilutions were used to measure ATP content with luciferase-based kit according to manufacturer instructions (MBL; ApoSENSOR kit). Results were normalized to protein content.

**Microscopy.** Transmission electron microscopy (TEM) analysis of *C. elegans* was done as described in ref. 31. DIC and immunofluorescence images were taken either with an Axiocam CCD camera mounted on a Zeiss Axioplan II microscope equipped for fluorescence and DIC, or with an MRC-1024 BioRad confocal scanhead coupled to a Zeiss Axiovert 135M inverted microscope equipped with a 63 $\times$  NA = 1.3 oil-immersion objective.

**RNA Isolation, Microarray Analysis, and Quantitative RT-PCR.** Wild-type (N2) *C. elegans* were grown for 1, 6 or 72 h in 19% CO<sub>2</sub> at 20 °C. All microarray experiments were performed in a pairwise manner. For the 1 and 6 h of exposure animals were grown at normal conditions until L4 and only then were transferred to 19% CO<sub>2</sub> for either 1 or 6 h, whereas control animals kept growing for either 1 or 6 h in air. For the 72 h of exposure, animals were grown from embryos until they reached the young adult stage, which takes between 72 and 80 h under 19% CO<sub>2</sub> and the developmental stage was determined using DIC microscopy. The control animals for that experiment were collected

at the same developmental stage. RNA preparations were isolated using trireagent and were used to synthesize first strand cDNA according to manufacturer instructions or to perform RT-qPCR. The RNA preparations were hybridized to *C. elegans* affymetrix gene array according to manufacturer's instructions. Real-time RT-PCR was performed on 2 independent isolations of RNA from each time point, which were different from the RNA isolation used for the microarray analysis. A Corbett ROTOR-GENE 6000 instrument using an ABgene ABSOLUTE qPCR SYBR green kit were used according to the manufacturer's instructions. *act-1* was used as the gene of reference.

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